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# Differential effects of age on chicken heterophil functional activation by recombinant chicken interleukin-2

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### Abstract

Interleukin-2 (IL-2) exercises an array of biological effects on many cells including the functional activation of cells of the innate immune response. Heterophils, the avian equivalent of the neutrophil, function as professional phagocytes to aid in regulation of innate host defenses. The objective of the present studies was to examine the effects of recombinant chicken IL-2 (rChIL-2) on functional activities of heterophils from chickens during the first 3 weeks after hatch. Peripheral blood heterophils were isolated and incubated with either COS cell-derived rChIL-2 or supernatants from mock-transfected COS cells. rChIL-2 had no effect on the functional activities of heterophils from day-of-hatch chickens, but significantly increased the phagocytosis and bactericidal activity of heterophils from 7- and 14-day-old chickens. rChIL-2 induced no direct stimulation of the respiratory burst by heterophils, but primed heterophils from 7- and 14-day-old birds for an enhanced respiratory burst in response to phorbol ester stimulation. Lastly, rChIL-2 had neither direct nor priming effects on heterophil degranulation. The enhancing effects on heterophil functional activity by rChIL-2 were abated by a neutralizing anti-chicken IL-2 mAb and were therefore specific for this cytokine. These results show that rChIL-2 can directly activate chicken heterophils to exert effector functions, and that heterophil activation by rChIL-2 is also an age-dependent event. © 2002 Elsevier Science Ltd. All rights reserved.

**Keywords:** Neutrophils; Cytokines; Cellular activation; Phagocytosis; Inflammation

### 1. Introduction

The common characteristic of both the innate and acquired immune systems is their ability to regulate their function through the prompt production of cytokines.

Heterophils are the primary effector cells of the acute innate host defenses to bacterial infections in poultry. Studies have shown a direct relationship between the high susceptibility of neonatal poultry

to infectious diseases and the functional immaturity of poultry heterophils during the first week post-hatch [1–5]. Likewise, the avian immune cells produce few or no cytokines during the first week post-hatch, further increasing the susceptibility of domestic poultry to infections [4]. Because of this deficiency in the functional ontogeny of the avian innate and acquired immune responses, there is a probable veterinary application for potentiating avian host defenses during the first week of life.

Cytokines are vital constituents in the regulation of immunity and inflammation [5]. The use of cytokines and cytokine genes as vaccine adjuvants,

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immunotherapeutics, and immune response modulators for treatment or prevention of cancer and infectious diseases are important focal points of current clinical research in both the human and veterinary medicine [6–10]. IL-2 is one such cytokine that is being investigated in both tumor and infectious disease immunology because it plays an active role in the activation and maintenance of both acquired and innate immune defenses [11–23]. Classically, IL-2 has been shown to be mitogenic for T lymphocytes, but has subsequently been found to stimulate growth-promoting activity in B cells and NK cells [11–14]. More recently, IL-2 has been shown to activate human monocytes and neutrophils to generate tumoricidal and microbicidal activities, produce an oxidative burst, and secrete several cytokines and bioactive lipids [15–23].

The recent cloning and sequencing of the avian IL-2 homologue has stimulated discussion on the use of this regulatory cytokine as either a vaccine adjuvant or immune modulating molecule in poultry [26,27]. Since the heterophil is a critical cell in the development of innate responses in poultry and is responsive to various cytokines, the objective of the present studies was to examine the effects of recombinant chicken IL-2 (rChIL-2) on the functional activation of heterophils from chickens during the first 3 weeks after hatch.

## 2. Materials and methods

### 2.1. Experimental animals

One-day-old Leghorn chicks (HyLine W-36<sup>®</sup>) were obtained from a commercial hatchery and randomly placed in electrically heated commercial brooder batteries (Petersime Incubator Co., Gettysburg, OH) located within a biological hazard isolation unit on the research farm of the College of Veterinary Medicine, Texas A&M University. Chicks were provided ad libitum access to water and a balanced unmedicated corn–soybean ration. The feed ration contained or exceeded the levels of critical nutrients recommended by the National Research Council [24].

### 2.2. Bacteria

A poultry isolate of *S. enteritidis* (SE), obtained from the National Veterinary Services Laboratory, Ames, IA, was used in the phagocytosis assays. A stock culture of SE was prepared in sterile phosphate-buffered saline and adjusted to a concentration of  $10^9$  cfu/ml using a spectrophotometer with a 625 nm reference wavelength.

### 2.3. Heterophil agonists

Phorbol myristate acetate (PMA; 1 mg/ml) was dissolved in dimethyl sulfoxide (DMSO) and stored at  $-20^{\circ}\text{C}$  as a stock solution until used in the experiments. The final concentration of DMSO in the assay media did not exceed 0.5% (v/v). Opsonized zymosan (OZ) was prepared by boiling zymosan A in 0.9% saline for 20 min and washing twice with saline. The unopsonized zymosan (20 mg/ml) was suspended in pooled normal chicken serum, opsonized for 30 min at  $39^{\circ}\text{C}$  on a rotary shaker, washed twice with  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ -free HBSS, and stored at  $4^{\circ}\text{C}$  in HBSS (20 mg/ml) until used.

### 2.4. Isolation of peripheral blood heterophils

Heterophils were isolated from the peripheral blood of Leghorn chickens as described previously [25]. Briefly, disodium ethylenediaminetetraacetic acid (EDTA)-anti-coagulated blood was mixed with 1% methylcellulose (25 cp; Sigma Chemical Co., St Louis, MO) as a 1.5:1 ratio and centrifuged at 25g for 5 min. The serum and buffy coat layers were retained and suspended in  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ -free Hanks' balanced salt solution (HBSS, 1:1; Sigma Chemical Co., St Louis, MO). This suspension was layered over a discontinuous Ficoll-Hypaque gradient (specific gravity 1.077 over specific gravity 1.119; Sigma Chemical Co., St Louis, MO). The gradient was then centrifuged at 250g for 20–25 min. After centrifugation, the 1.077/1.119 interface and 1.119 band containing the heterophils were collected and washed twice in RPMI 1640 medium and resuspended in fresh RPMI 1640. Cell viability was determined by trypan blue exclusion. The purity of the heterophil suspensions was assessed by microscopic examination of Hema-3 stained (Curtin Mathison Scientific, Dallas, TX)

cytospin (Shandon Scientific, Pittsburgh, PA) smears. Heterophil preparations obtained by this method were typically >95% pure and >95% viable. The cell concentration was adjusted to  $4 \times 10^6$  heterophils/ml and stored on ice until used.

### 2.5. Production of recombinant ChIL-2

Supernatants of COS cells containing rChIL-2 were produced as described [26]. Briefly, primers were designed to ChIL-2 cDNA clones isolated by Sundick and Gill-Dixon [27]. COS-7 cells were routinely grown in DMEM containing 10% FCS, 1% non-essential amino acids, 1% L-glutamine, 1 U/ml penicillin, and 1  $\mu$ g/ml streptomycin at 37 °C, 5% CO<sub>2</sub>, and passaged using standard conditions [26]. Cells were cultured at  $5 \times 10^5$  ml<sup>-1</sup> for 18–24 h at 37 °C, 5% CO<sub>2</sub>, and washed twice with PBS. Five milliliters of serum-free media was then added containing 7.5  $\mu$ g/ml DNA (pCIneo containing rChIL-2 or no plasmid), 258  $\mu$ g/ml chloroquine and 600  $\mu$ g/ml DEAE-dextran. Flasks were incubated for 3 h at 37 °C, 5% CO<sub>2</sub>. The transfection media was then removed and cells washed once with PBS. PBS containing 10% dimethylsulfoxide was then added for 2 min, removed and replaced with 5 ml growth medium. After 24 h growth at 37 °C, 5% CO<sub>2</sub>, growth media was replaced with serum-free DMEM. The cells were then incubated for 72 h, following which supernatant was collected and stored at 4 °C prior to use in the assays.

### 2.6. Experimental design

Chicken heterophils ( $4 \times 10^6$  ml<sup>-1</sup>) were incubated with various concentrations of COS cell-derived rChIL-2 for 2 h at 39 °C on a rotary shaker in a 5% CO<sub>2</sub> incubator. The heterophils were then used to perform the functional assays described below. All functional assays were conducted at least three times and triplicate readings were recorded for each assay. Control assays were performed using heterophils incubated with mock-transfected COS cell supernatants.

The anti-coagulated blood from 50 chickens was pooled and the heterophils were isolated from each treatment group as described above. Each heterophil functional assay was conducted four times over a

2-month period with pooled heterophils (heterophils pooled from 50 chickens for each preparation; i.e. 200 total chickens were used as cell donors). At least three replicates were conducted for each heterophil functional assay with the heterophils from each pool of chickens. The data from these four repeated experiments were pooled for presentation and statistical analysis.

### 2.7. mAb neutralization of rChIL-2

To confirm the specificity of the rChIL-2-inducing activities, the rChIL-2 was preincubated (1:1) with mouse anti-rChIL-2 neutralizing mAb (4F12) [28] on a rocker platform for 1 h at room temperature. After this incubation, the suspension was filtered through a YM100 ultrafiltration membrane (Amicon Corporation, Danvers, MA) to remove any rChIL-2-antibody complexes and/or unbound IgG molecules before use in any assays. As controls, rChIL-2 was also pretreated with a preimmune mouse IgG as described for the anti-rChIL-2.

### 2.8. Phagocytosis assay

Phagocytosis of unopsonized SE by the heterophils was determined by using duplicate, sterile 15 ml polypropylene screw-capped centrifuge tubes that contained  $2 \times 10^6$  heterophils (500  $\mu$ l) and  $2 \times 10^7$  bacteria (200  $\mu$ l) in a total volume of 1 ml RPMI 1640 as described [25]. The tubes were centrifuged (450g, 15 min, room temperature) in order to maximize contact between heterophils and bacteria. The cells were then allowed to incubate at 39 °C for 30 min on a rotary shaker to allow for phagocytosis. After each incubation period, the tubes were placed on ice to stop phagocytosis. The cells were washed three times with ice-cold RPMI 1640, centrifuged, and resuspended in 1 ml cold RPMI 1640. Cytospin smears were then prepared from each tube, stained with Hema-3, and examined by light microscopy with the oil immersion objective (100 $\times$ ).

An individual unaware of the groups performed all counts. At least 100 heterophils on each slide were scored for the percentage of heterophils that contained bacteria and for the number of bacteria associated with each heterophil. The results are expressed as the percentage of heterophils that contain bacteria, the

average number of bacteria per ingesting heterophil, and the phagocytic index (PI), where  $PI = (\text{percentage of heterophils that contain bacteria} \times \text{the average number of bacteria per ingesting heterophil}) \times 100$ .

### 2.9. Bactericidal assay

The bactericidal activity of peripheral blood heterophils in 96-well microtiter plates was evaluated as described previously [25]. Briefly,  $1 \times 10^6$  SE (100  $\mu\text{l}$ ) were added to quadruplicate wells containing  $2 \times 10^5$  heterophils (100- $\mu\text{l}$  of  $2 \times 10^6$  cells/ml suspension). Control wells contained HBSS alone. In addition, a suspension of  $2 \times 10^7$  bacteria (100%) in HBSS was diluted to 70, 40, and 10% of this concentration, and 100- $\mu\text{l}$  amounts of each dilution were added to quadruplicate wells for the construction of a standard curve. The plates were centrifuged (400g for 5 min at 4 °C) to allow maximum bacteria/heterophil contact. At this moment, one plate was removed, the heterophils lysed, and the number of bacteria determined using the XTT assay as described below. This was considered to be the time = 0 time point. The remaining plates were then incubated for either 30 or 60 min at 37 °C. After this incubation, the heterophils were lysed by adding 50  $\mu\text{l}$  of 0.1% Triton X-100. Fifty microliters of 3,3'-[1[(phenylamino)carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT, 0.5 mg/ml; Sigma Chemical Co., St Louis, MO) containing 40  $\mu\text{g/ml}$  of 2,3-dimethoxy-5-methyl-1,4-benzoquinone (coenzyme Q, CQ; Sigma Chemical Co., St Louis, MO) were added to each well and the plates were returned to the incubator for 30 min to allow the viable bacteria to reduce the XTT to formazan. The soluble formazan produced by the bacteria was then quantitated by measuring the optical densities of each well at 450 nm with an automated ELISA plate reader (Bio-Tek, Model EL 311, Winooski, VT). Bactericidal activity of the test wells was extrapolated from a standard curve generated using known bacterial concentrations. Each plate was compared to its own standard curve to account for day-to-day and plate-to-plate variations. The standard curve was verified by standard plate counts. The results are expressed as the percent bacterial killing.

### 2.10. Oxidative burst

The oxidative burst of the rChIL-2-treated chicken heterophils was measured by luminol-dependent chemiluminescence (LDCL). One hundred microliters of heterophils ( $4 \times 10^6$  cells/ml), 100  $\mu\text{l}$  of OZ (20 mg/ml), and 500  $\mu\text{l}$  of luminol (0.1 M in RPMI 1640) were placed in each Beckman polypropylene scintillation vial and incubated at room temperature for 30 min. The LDCL was measured in a LKB 1219 series liquid scintillation counter using the tritium channel and the coincidence mode. LDCL was quantified as cpm. All samples were assayed in replicates of five vials. Results are expressed as cpm/ $10^6$  heterophils.

### 2.11. Degranulation

Degranulation was detected by quantifying the amount of  $\beta$ -D-glucuronidase activity in the culture medium following stimulation of the heterophils with OZ. Heterophils ( $8 \times 10^6 \text{ ml}^{-1}$ ) were pre-treated with either rChIL-2 or supernatants from mock-transfected COS cells and then incubated with OZ (2 mg/ml) for 1 h at 39 °C on a rotary shaker in a 5%  $\text{CO}_2$  incubator. The reaction was stopped by transferring the tubes containing the cells to an ice bath for 5–10 min. The cells were then centrifuged at 250g for 10 min at 4 °C. The supernatants were then removed and used for the assay. Samples (25  $\mu\text{l}$ ) of each supernatant were added to quadruplicate wells in a non-treated black CoStar flat-bottom ELISA plate and incubated with 50  $\mu\text{l}$  of freshly prepared substrate (10 mM 4-methylumbelliferyl- $\beta$ -D-glucuronide, 0.1% Triton X-100 in 0.1 M sodium acetate buffer) for 4 h at 41 °C. The reaction was stopped by adding 200  $\mu\text{l}$  of a stop solution (0.05 M glycine and 5 mM EDTA; pH 10.4). Liberated 4-methylumbelliferone was measured fluorimetrically (excitation wavelength of 355 nm and an emission wavelength of 460 nm) with a  $f_{\text{max}}$  fluorescence microplate reader (Molecular Devices, Sunnyvale, CA). These values were converted to nanomoles of 4-methylumbelliferone generated using a standard curve of known concentrations of 4-methylumbelliferone.

Table 1  
Effect of recombinant chicken IL-2 on phagocytosis of *Salmonella enteritidis* (SE) by heterophils isolated from chicks at different ages

Age	Dilution of rChIL-2 (log 10)			Percentage of heterophils containing SE					Average number of SE per ingesting heterophil					Phagocytic index				
	0 <sup>a</sup>	100	1000	10,000	0 <sup>a</sup>	100	1000	10,000	0 <sup>a</sup>	100	1000	10,000	0 <sup>a</sup>	100	1000	10,000	0 <sup>a</sup>	10,000
1	26.8 ± 2.2	24.5 ± 2.0	23.4 ± 2.7	25.5 ± 1.8	2.16 ± 0.58	2.21 ± 0.55	2.33 ± 0.34	2.60 ± 0.41	57.89	54.15	54.52	66.30	57.89	54.15	54.52	66.30	57.89	66.30
7	32.3 ± 1.3	41.8 ± 3.1*	37.5 ± 3.6*	31.2 ± 2.2	2.22 ± 0.23	3.42 ± 0.69*	2.77 ± 0.51*	2.25 ± 0.44	71.71	142.96*	103.88*	70.20	71.71	142.96*	103.88*	70.20	71.71	70.20
14	44.0 ± 3.6	54.8 ± 3.4*	55.2 ± 3.1*	48.2 ± 2.9*	3.72 ± 0.54	4.23 ± 0.85*	5.12 ± 0.62*	4.47 ± 0.74*	163.68	231.80*	282.62*	215.45*	163.68	231.80*	282.62*	215.45*	163.68	215.45*

Within rows, numbers with \* are significantly different from their age-matched control heterophils.

<sup>a</sup> Mock transfectant control (1:100 dilution).

## 2.12. Statistical analysis

The mean and standard error of the mean were calculated for each of the treatment groups in each of the heterophil functional assays. Functional differences between the rChIL-2-treated and the mock transfectant control supernatant-treated control cells were determined by analysis of variance. Significant differences were further separated using Duncan's multiple range test. The data are presented in all figures and tables as mean ± SEM.

## 3. Results

### 3.1. Phagocytosis

The ability of avian heterophils preincubated with rChIL-2 to phagocytize non-opsonized SE was determined and the experiments are summarized in Table 1. ChIL-2 had no effect on phagocytosis by heterophils isolated from day-of-hatch chicks. Heterophils isolated from chicks 7- and 14-days post-hatch primed with the 1:100 or 1:1000 dilution of ChIL-2 exhibited significantly ( $p < 0.01$ ) greater phagocytic activity as compared to the cells treated with the supernatants from the mock-transfected COS cells. This phagocytic activity was concentration-dependent and manifested by both an increase in the percentage of heterophils that phagocytized SE and an increase in the number of bacteria that were engulfed by each heterophil.

### 3.2. Bacterial killing

The effects of rChIL-2 on the bactericidal activity of heterophils isolated from chicks at day-of-hatch, 7- or 14-days after hatch are shown in Fig. 1. rChIL-2 pretreatment had no significant effect on the bactericidal activity of heterophils from the day-of-hatch chicks increasing the killing activity of the cells by less than 5% regardless of the concentration of cytokine used. However, rChIL-2 pretreatment of the heterophils from 7- and 14-day-old chicks induced a significant ( $p < 0.01$ ) concentration-dependent increase in bacterial killing ability. Depending upon concentration of the cytokine, bactericidal activity



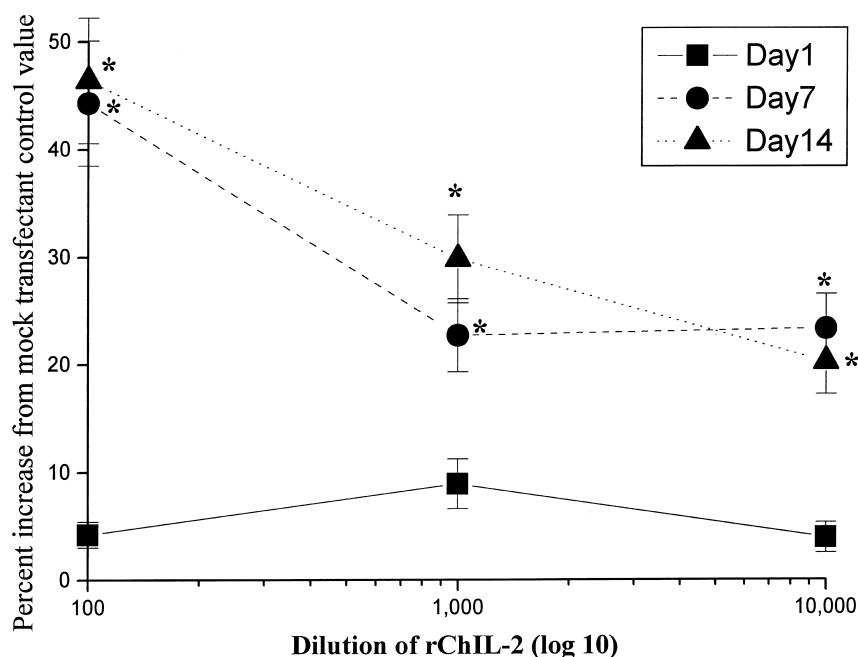


Fig. 1. Bactericidal activity of heterophils isolated from chickens at 1-, 7-, and 14-days post-hatch. Heterophils were pretreated with various dilutions of COS cell-derived rChIL-2 for 2 h 39 °C as described in Section 2. The data points with asterisks indicate a significant increase in heterophil bactericidal activity, when compared to the age-matched heterophils pretreated with mock-transfected COS cell supernatants. Data presented as mean  $\pm$  SEM.

was increased from 22 to 50% over the supernatants from the mock transfectant-treated heterophils.

### 3.3. Oxidative burst

The effects of rChIL-2 on the oxidative burst generated by heterophils isolated from chicks at different days post-hatch are shown in Table 2.

Treatment of the heterophils with the rChIL-2 did not directly stimulate an oxidative burst. Likewise, rChIL-2 treatment of heterophils from day-of-hatch chicks had no priming effect on the PMA-induced oxidative burst. However, treatment of heterophils from 7- and 14-day old chicks with rChIL-2 primed the cells for at least a 2-fold increase ( $p < 0.01$ ) in PMA-induced oxidative burst as compared to the

Table 2  
Effect of recombinant chicken IL-2 on oxidative burst generated by heterophils isolated from chicks at different ages

Age (days)	PMA <sup>a</sup>	Chemiluminescence; peak cpm ( $\times 10^6$ ); dilution of rChIL-2 (log 10)			
		0 <sup>b</sup>	100	1000	10,000
1	–	0.49 $\pm$ 0.11	0.51 $\pm$ 0.09	0.49 $\pm$ 0.10	0.48 $\pm$ 0.13
	+	9.9 $\pm$ 1.5	9.6 $\pm$ 1.8	9.9 $\pm$ 1.9	8.3 $\pm$ 1.2
7	–	2.30 $\pm$ 0.62	2.05 $\pm$ 0.43	1.83 $\pm$ 0.44	2.22 $\pm$ 0.31
	+	14.4 $\pm$ 1.7	29.1 $\pm$ 3.0*	24.3 $\pm$ 2.2*	12.6 $\pm$ 2.1
14	–	3.41 $\pm$ 0.33	3.2 $\pm$ 0.3	3.9 $\pm$ 0.2	4.1 $\pm$ 0.6
	+	14.7 $\pm$ 2.2	36.8 $\pm$ 4.4*	39.6 $\pm$ 3.4*	18.9 $\pm$ 1.3

Within rows, numbers with \* are significantly different from their age-matched control heterophils.

<sup>a</sup> Heterophils either non-stimulated or stimulated with 2  $\mu$ g/ml PMA.

<sup>b</sup> Mock transfectant control (1:100 dilution).

Table 3  
Effect of recombinant chicken IL-2 on degranulation by heterophils isolated from chicks at different ages

Age (days)	OZ <sup>a</sup>	$\mu\text{M } \beta\text{-D-glucuronidase released; dilution of rChIL-2 (log 10)}$			
		0 <sup>b</sup>	100	1000	10,000
1	–	1.83 $\pm$ 0.32	1.76 $\pm$ 0.39	1.72 $\pm$ 0.67	1.56 $\pm$ 0.41
	+	12.91 $\pm$ 2.12	11.98 $\pm$ 1.83	12.11 $\pm$ 2.17	12.05 $\pm$ 1.92
7	–	2.64 $\pm$ 0.77	2.80 $\pm$ 0.48	2.76 $\pm$ 0.51	2.56 $\pm$ 0.87
	+	12.21 $\pm$ 1.98	12.05 $\pm$ 2.11	12.47 $\pm$ 1.23	13.43 $\pm$ 2.76
14	–	6.53 $\pm$ 2.19	6.55 $\pm$ 1.78	7.13 $\pm$ 1.69	6.77 $\pm$ 1.49
	+	16.44 $\pm$ 3.12	15.23 $\pm$ 1.11	14.59 $\pm$ 1.77	13.14 $\pm$ 2.21

Within rows, numbers with \* are significantly different from their age-matched control heterophils.

<sup>a</sup> Heterophils either non-stimulated or stimulated with 2 mg/ml OZ.

<sup>b</sup> Mock transfectant control (1:100 dilution).

age-matched control heterophils treated with supernatants from the mock-transfected COS cells.

### 3.4. Degranulation

Treatment of heterophils from any age chicks with either rChIL-2 or supernatants from the mock transfected COS cells did not induce the direct release of the primary granule,  $\beta\text{-D-glucuronidase}$  (Table 3). Interestingly, treatment of the heterophils isolated from any age chick with rChIL-2 did not induce a significantly different priming response to OZ as compared to the supernatants from the mock-transfected COS cells.

### 3.5. mAb neutralization—phagocytosis

Treatment of rChIL-2 (1:100 dilution of stock supernatant) with 4F12 specifically neutralized the rChIL-2-mediated increase in phagocytosis by heterophils isolated from 7- and 14-day-old chicks (Fig. 2A and B). Pretreatment of the rChIL-2 with the control murine IgG had no neutralizing activity on phagocytosis.

### 3.6. mAb neutralization—bacterial killing

Treatment of rChIL-2 with 4F12 specifically neutralized all of the rChIL-2-mediated increase in bactericidal activity by heterophils from either 7- or 14-day-old chicks (Fig. 3A and B). Pretreatment of the rChIL-2 with the control murine IgG had no

neutralizing action on the killing activity by the heterophils.

### 3.7. mAb neutralization—oxidative burst

The monoclonal antibody, 4F12, specifically neutralized all of the rChIL-2-mediated priming of the PMA-induced oxidative burst by the heterophils from 7- and 14-day-old chicks (Fig. 4A and B) while the control murine IgG had no neutralizing effect on heterophil priming.

## 4. Discussion

We have previously demonstrated that undefined T cell supernatants can enhance the functional capabilities of these neonatal poultry heterophils to mimic the function of heterophils from mature birds [2,25,28]. Furthermore, we have shown a correlation between the enhancement of heterophil function in vitro with an increase in host resistance to *Salmonella* infections in vivo [25]. We also found that rChIFN- $\gamma$  is capable of enhancing the functional efficiency of heterophils from neonatal chickens [30].

Long thought to be a growth factor primarily involved in the activation of B and T lymphocytes [12,13,30,31], IL-2 has proven to be a much more pleiotropic cytokine. Evidence from recent years has shown that IL-2 plays a critical role in cells specifically involved in the innate immune responses including NK cells, monocytes, eosinophils, and neutrophils [14–23]. Furthermore, IL-2 has been shown to modulate the



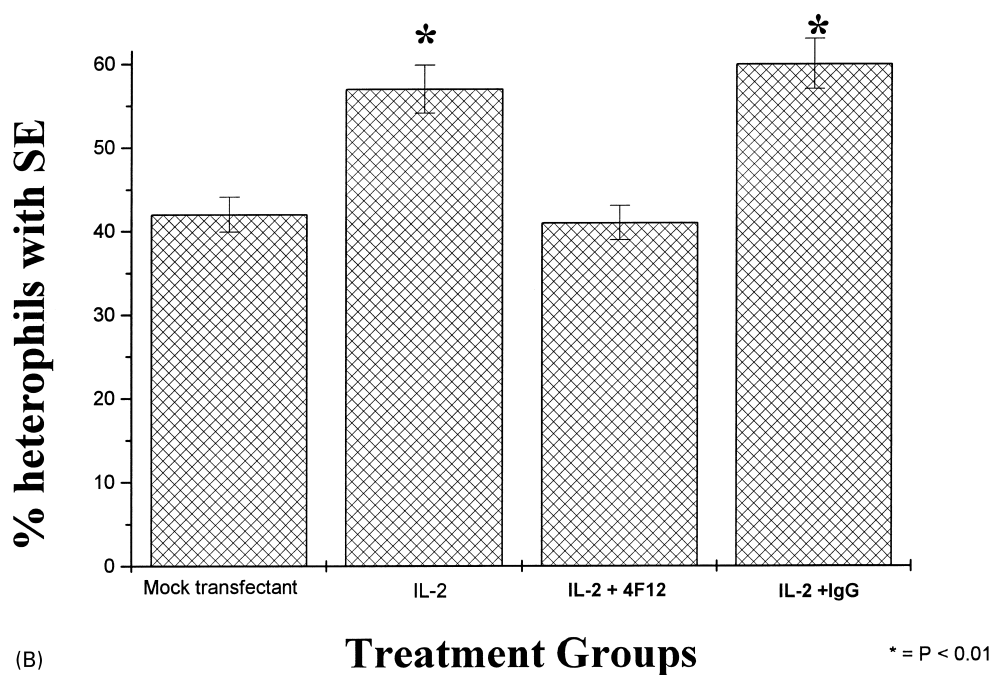
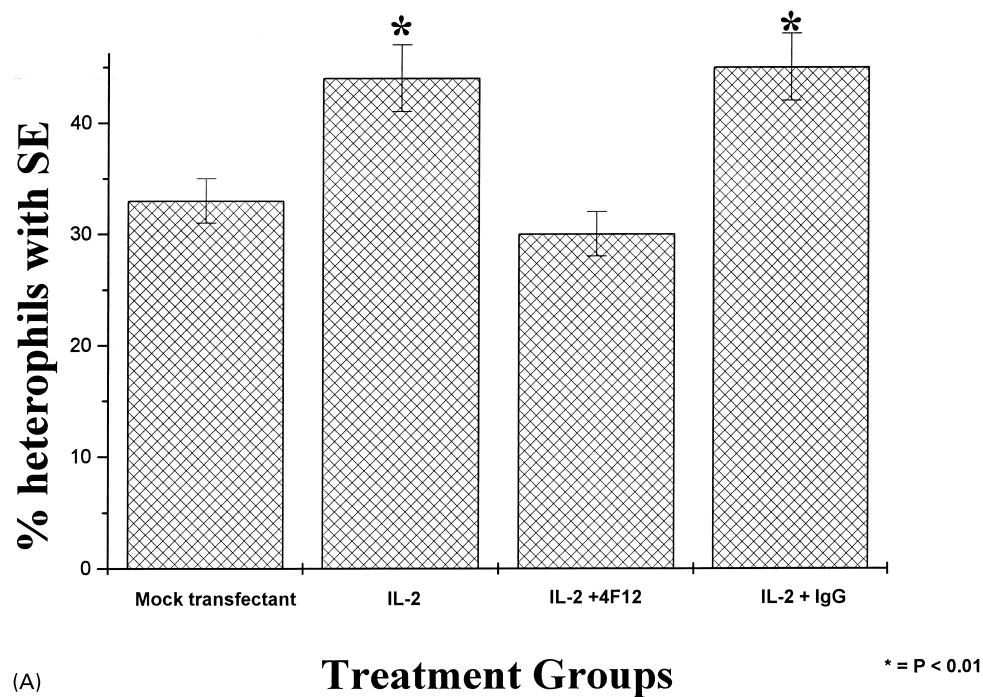


Fig. 2. Effect of mAb (4F12) pretreatment of COS-cell-derived rChIL-2 on phagocytosis of non-opsonized SE by heterophils isolated from chickens 7- and 14-days post-hatch (A and B, respectively). The COS cell-derived rChIL-2 was pretreated with 4F12 as described in Section 2. Bars with asterisks indicate a significant increase in phagocytosis from age-matched heterophils incubated for 2 h with 4F12-treated rChIL-2. Data presented as mean  $\pm$  SEM.

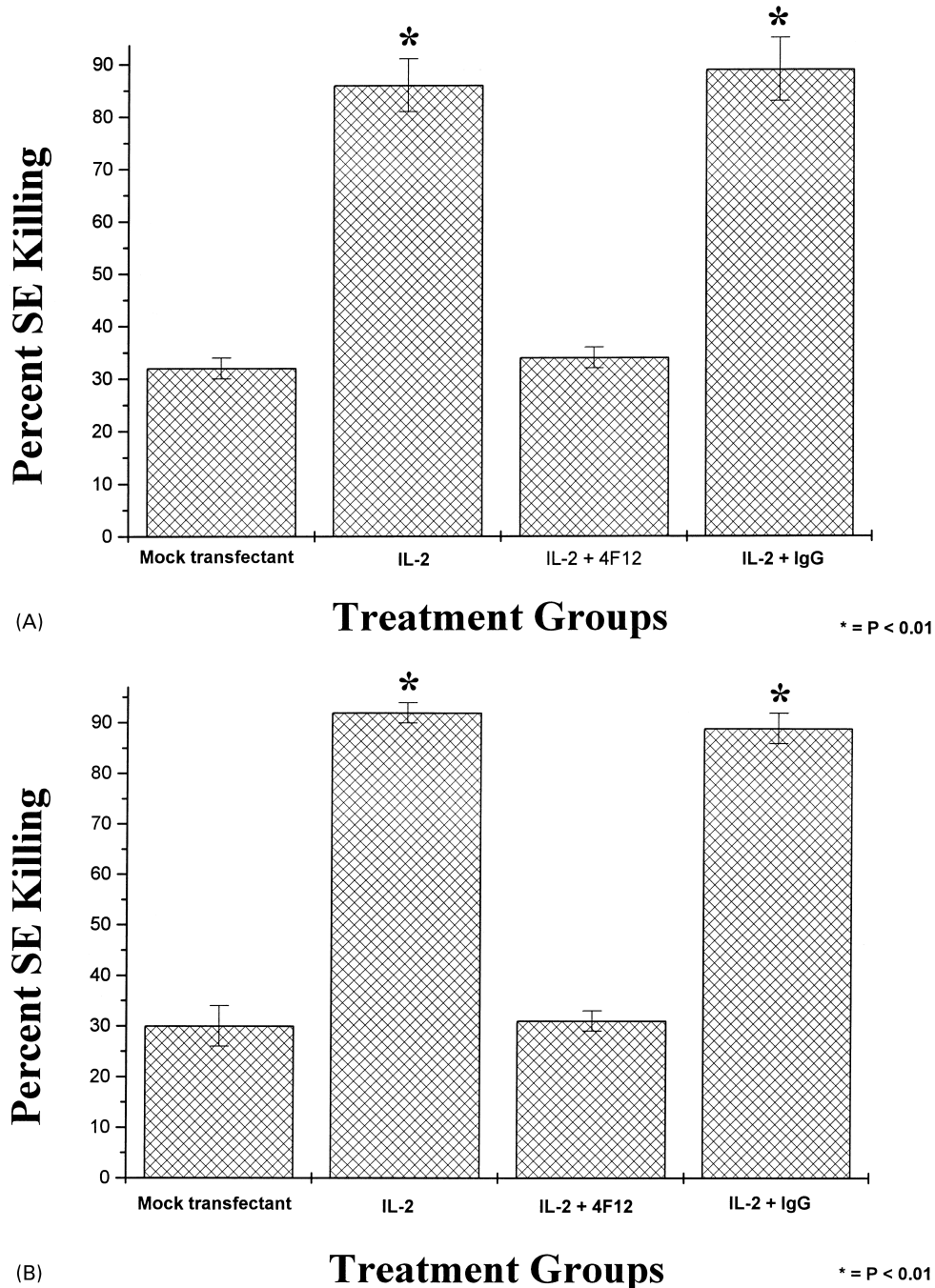


Fig. 3. Effect of mAb (4F12) pretreatment of COS cell-derived rChIL-2 on the bactericidal activity of heterophils isolated from chickens 7- or 14-days post-hatch (A and B, respectively). The COS cell-derived rChIL-2 was pretreated with 4F12 as described in Section 2. Bars with asterisks indicate a significant increase in bactericidal activity from age-matched heterophils incubated for 2 h with 4F12-treated rChIL-2. Data presented as mean  $\pm$  SEM.

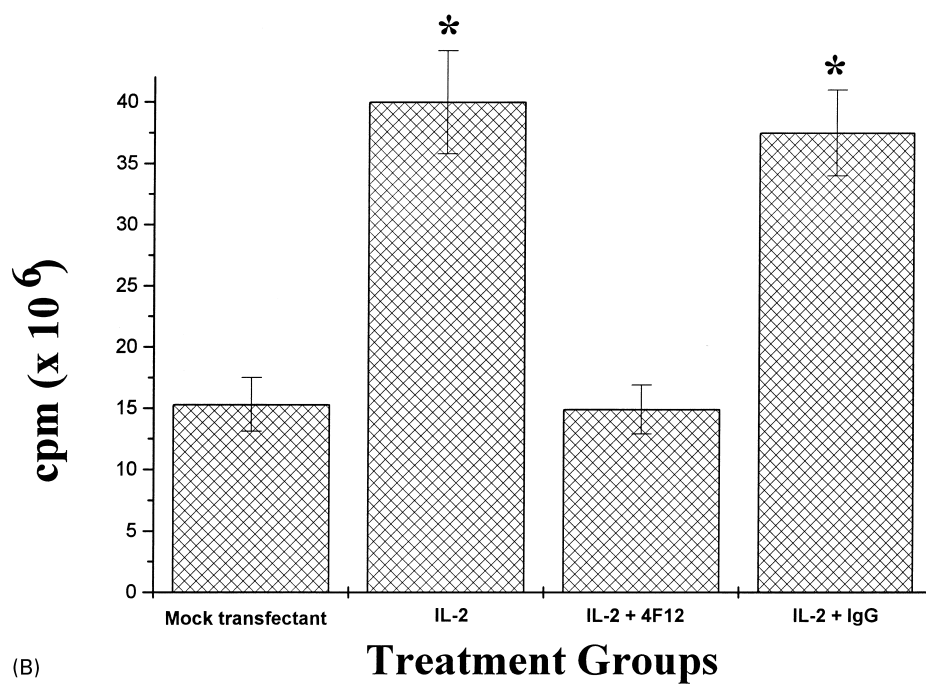
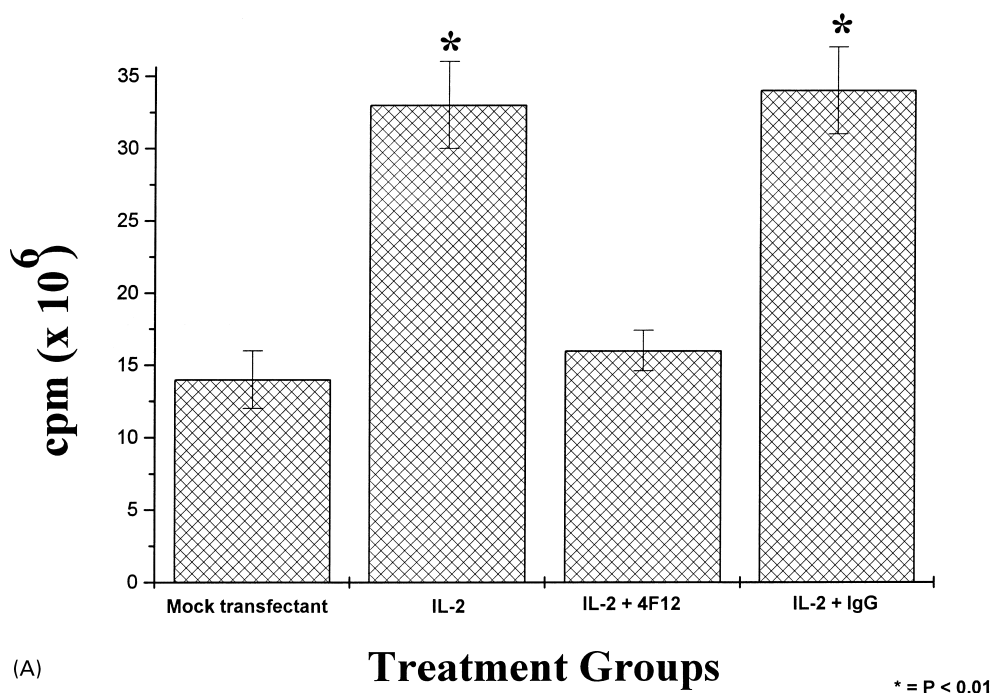


Fig. 4. Effect of mAb (4F12) pretreatment of COS cell-derived rChIL-2 on the respiratory burst of heterophils isolated from chickens 7- or 14-days post-hatch (A and B, respectively). The COS cell-derived rChIL-2 was pretreated with 4F12 as described in Section 2. Bars with asterisks indicate a statistically significant increase in respiratory burst from age-matched heterophils incubated for 2 h with 4F12-treated rChIL-2. Data presented as mean  $\pm$  SEM.

function of intestinal epithelial cells [32,33]. Here, we provide evidence that IL-2 from chickens can upregulate certain functions of the heterophil.

IL-2 binds to specific cell receptors. In mammals, IL-2 receptors (IL-2R) are composed of at least three subunits,  $\alpha$ -,  $\beta$ -, and  $\gamma$ -chains [34,35]. The  $\beta$  and  $\gamma$  chains have intermediate binding affinity and mediate many of the biological activities of IL-2. IL-2R $\beta$  and IL-2R $\gamma$  are constitutively expressed on neutrophils [15,16]; whereas, IL-2R $\alpha$  is absent [15]. The avian IL-2R has not been well characterized, although a mAb thought to recognize the avian IL-2R  $\alpha$ -chain has been developed [36,37]. Using this mAb against the avian IL-2R $\alpha$  chain, we could not block the functional activation of heterophils induced by the rChIL-2 (data not shown). However, an anti-rChIL-2R mAb neutralized IL-2-mediated functional activation. It is therefore reasonable to speculate that the avian heterophil may also constitutively express IL-2R $\beta$  and  $\gamma$ . However, until more tools are developed for chicken IL-2 receptors, this remains speculation on our part.

In this study, rChIL-2 significantly increased phagocytic activity in avian heterophils when compared to the effect of mock-transfected cell supernatants (Table 1). Definitive proof that IL-2 was inducing the enhanced phagocytosis was obtained by the ability of the specific anti-ChIL-2 mAb to abrogate the phagocytic activity of the cells. These results differ from those observed with IL-2-treated mammalian neutrophils where the cytokine had no effect on the phagocytic activity of these granulocytic cells [38, 39]. While at this time we have no explanation for the apparent difference in IL-2-mediated enhancement of phagocytosis between mammalian neutrophils and avian heterophils, it can be pointed out that our phagocytosis experiments were conducted without opsonizing the bacteria. Therefore, we can eliminate both Fc and complement receptor involvement in the IL-2-mediated increase in phagocytosis since one mechanism of phagocytosis is triggered through receptors for opsonized particles. In contrast, opsonin-independent phagocytosis, as described here, is mediated by receptors on the plasma membranes of the phagocyte that directly recognize ligands on the surfaces of the microbe [40]. These cellular receptors are members of a family of germ-line encoded molecules titled pattern recognition receptors

(PRRs) that recognize conserved motifs (pathogen recognition molecular patterns, PAMPs) on the microbial pathogens that are not found in eukaryotes [41]. These PRRs include surface molecules such as mannose and scavenger receptors [42–44]. Both mannose and scavenger receptor expression and activity are regulated by cytokines [45–47]. It is possible that the IL-2-mediated increase in phagocytosis observed in these studies resulted from an increased expression and/or activity of mannose or scavenger receptors. Further experiments are presently underway to confirm this hypothesis.

Following phagocytosis, we demonstrated that treatment of the heterophils with rChIL-2 enhanced the bactericidal activity of the cells (Fig. 3) and that this activity was also neutralized by the anti-ChIL-2mAb. Two bactericidal processes are available to the heterophil: the oxidative burst or the release of lytic enzymes and anti-microbial peptides from intracellular granules (degranulation). Analysis of the mechanism of bacterial killing by IL-2-stimulated heterophils indicated that degranulation was not involved as shown by both the lack of  $\beta$ -D-glucuronidase release following by IL-2 treatment and the inability of IL-2 to prime the heterophil for  $\beta$ -D-glucuronidase release by zymosan stimulation. In contrast, IL-2 was a potent primer of the heterophils for an increased oxidative burst upon stimulation with PMA (Tables 2 and 3), which was inhibited following treatment with the anti-rChIL-2 mAb, 4F12 (Fig. 4). IL-2 had no direct stimulatory activity on the heterophil oxidative burst. These results are in direct contrast to those found with IL-2 treatment of mammalian neutrophils where IL-2 had no effect on the generation of an oxidative burst [39], but primes the cells for increased release of the granule lactoferrin [15]. It remains to be determined why the response of avian heterophils to IL-2 is different than the response of mammalian neutrophils. We have shown previously that rChIFN- $\gamma$  is a potent priming agent for both anti-microbial killing mechanisms of avian heterophils [30]. Interestingly, we found that rChIFN- $\gamma$  is capable of enhancing the functional efficiency of heterophils from day-old chickens. These results were in agreement with mammalian studies that demonstrated that cytokines have a more profound effect on neutrophils with depressed function than on normal neutrophils [48]. Taken together, the results from the present studies

suggest that IL-2 may play a very specialized role in a critical function of avian heterophils; i.e. oxygen-dependent anti-microbial activity, at a time post-hatch (by 7 days) that initiates the onset of measurable specific acquired immunity to vaccines given in ovo or immediately post-hatch [49]. We can speculate that IL-2, produced during the activation of T lymphocytes, could be functioning not only as a T cell growth factor, but also as a supplemental heterophil-activating agent. Therefore, we propose that the biological effects of IL-2 in birds could be extended to include a supporting role in heterophil-mediated innate immune responses.

The present report demonstrates that IL-2 plays a role in heterophil activation. The data define a new pathway where IL-2 can augment acute inflammation by supporting both phagocytosis and oxidative burst. Taken together with our previous reports that heterophils can respond to rChIFN- $\gamma$  and non-specific T-cell lymphokines with increased phagocytosis and bacterial killing [23,25,29,30], the data suggest that heterophils possess many cytokine receptors; and thus, are able to respond rapidly and efficiently to inflammatory mediators triggered by the invasion of mucosal surfaces by pathogenic microorganisms. Our data certainly support the view that the biologic effects of IL-2 extend beyond T- and B-cell growth promotion and activation to include a role in developing heterophil-mediated innate immunity.

This study does not address the question of the *in situ* source of IL-2. Although systemically produced IL-2 could possibly be involved in heterophil activation, we support the hypothesis that local production of IL-2 plays a more active role in supplementing heterophil activation during acute inflammation. For example, in response to pathogen invasion in the intestine, IL-2 is produced by subpopulations of T cells that reside within the intestinal epithelium of both mammals and chickens [50–56]. The IL-2 produced by these intraepithelial lymphocytes (IELs) promotes epithelial wound healing [51] and the growth and effector function differentiation of all IEL subpopulations [54,56]. Likewise, bacterial invasion of the mucosal epithelium stimulates the production of IL-8, which is responsible for the recruitment of granulocytic cells to the local site of inflammation [57,58]. In chickens, intestinal invasion by paratyphoid *Salmonella* stimu-

late a strong acute inflammatory response mediated by the differential expression and production of inflammatory cytokines and the migration of heterophils to the local site of invasion [59]. Therefore, both heterophils and IL-2 are present locally.

In summary, we have provided data that demonstrates ChIL-2 is capable of augmenting certain functional activities of chicken heterophils. This heterophil activation is an age-dependent event since heterophils from day-old chickens are not responsive to the ChIL-2. The effects of IL-2 on heterophils were specific because a neutralizing anti-ChIL-2 mAb completely inhibited the functional activation of the heterophils. These results illustrate that ChIL-2 is more pleiotropic than believed since it can directly activate chicken heterophils to exert effector functions.

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